

## Note

## Correspondence

Michael R. Enright  
michael.r.enright@may.ie

## Characterization of endospore-forming bacteria associated with entomopathogenic nematodes, *Heterorhabditis* spp., and description of *Paenibacillus nematophilus* sp. nov.

Michael R. Enright,<sup>1,2</sup> James O. McInerney<sup>2</sup> and Christine T. Griffin<sup>1,2</sup>

Institute of Bioengineering and Agroecology<sup>1</sup> and Department of Biology<sup>2</sup>, National University of Ireland, Maynooth, Co. Kildare, Ireland

Endospore-forming bacteria were isolated from insect-pathogenic nematodes, *Heterorhabditis* spp., from three diverse geographical locations. Spindle-shaped sporangia of these bacteria adhere to the free-living infective stage of the nematode, which carries them to new insect hosts, where the bacterium reproduces. These isolates were characterized based on phenotypic and chemotaxonomic properties and 16S rRNA gene sequences. Analysis of the 16S rRNA gene placed the isolates within the genus *Paenibacillus*. The isolates shared higher sequence similarities with each other (95.1–100%) than they did with any other named species within the genus (89.2–94%). *Paenibacillus macquariensis*, *Paenibacillus azoreducens*, *Paenibacillus amylo-lyticus* and *Paenibacillus durus* were among the species with highest sequence similarity to these isolates. The isolates shared a high degree of phenotypic similarity and were easily distinguished from closely related members of the genus. Anteiso-C<sub>15:0</sub> and C<sub>16:0</sub> were among the major fatty acid types and the DNA G + C content was approximately 44 mol% in all isolates. DNA–DNA similarity studies revealed genomic heterogeneity among the isolates, such that they are likely to represent more than one species. Two of the isolates (both from a *Heterorhabditis megidis* isolate from Estonia) are phenotypically distinguishable from the others and are proposed as a single species, *Paenibacillus nematophilus* sp. nov. The type strain for this novel species is NEM1a<sup>T</sup> (= DSM 13559<sup>T</sup> = NCIMB 13845<sup>T</sup>). The other isolates, although closely related to the proposed species, are likely to represent at least one, but most likely two, novel species.

The genus *Paenibacillus* includes several species that are associated with dead insects in one way or another. Amongst these are obligate insect pathogens such as *Paenibacillus popilliae*, *Paenibacillus lentimorbus* and *Paenibacillus larvae* (Pettersson *et al.*, 1999), as well as non-pathogens/facultative pathogens such as *Paenibacillus apiarus* (Nakamura, 1996) and *Paenibacillus alvei* (Krieg, 1981). Enright *et al.* (2001) found a *Paenibacillus* sp. associated with an insect-pathogenic nematode, *Heterorhabditis megidis* EU17. This nematode was isolated during a survey conducted in Estonia (Griffin *et al.*, 1999).

*Heterorhabditis* spp. nematodes are lethal pathogens of insects. Infective juveniles (IJs), the only infective stage of

these nematodes, carry in their intestine a symbiotic bacterium belonging to the genus *Photorhabdus* (Forst *et al.*, 1997). Upon infection of a suitable host insect, the symbiont is released into the haemocoel and causes the death of the insect within 48 h (Poinar, 1990). The *Photorhabdus* sp. then completely colonizes the dead insect cadaver, assisting in the breakdown of the insect tissues by secretion of an array of enzymes (Forst & Neilson, 1996). It is upon this bacterium and the degraded tissues that the nematodes feed. The nematodes complete their development and reproduce within the dead host. *Photorhabdus* spp. also produce several antibiotics (Forst & Neilson, 1996). Together, these have a broad spectrum of activity (Akhurst, 1982) and help to ensure the dominance of *Photorhabdus* within the insect cadaver (Forst *et al.*, 1997). Until its nutritive status declines, the insect cadaver supports several nematode generations. Thousands of the non-feeding IJs then emerge from the insect into the soil, in search of a new insect host (Poinar, 1990).

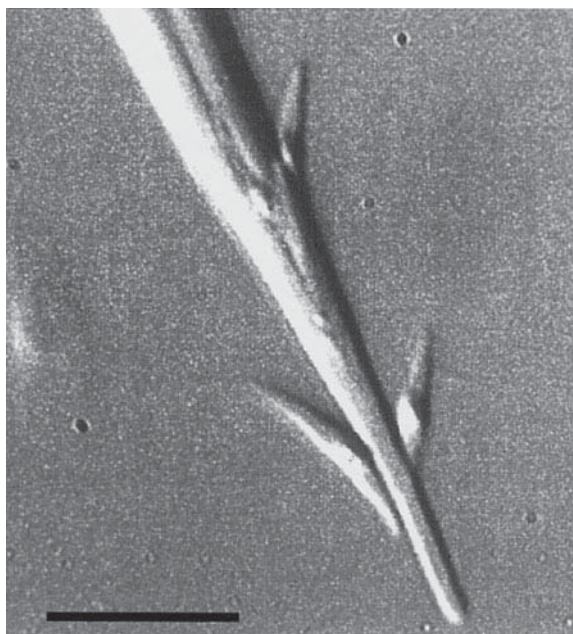
The association reported by Enright *et al.* (2001) involves spindle-shaped swollen sporangia of a *Paenibacillus* sp.

Abbreviation: IJ, infective juvenile.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strains NEM1a<sup>T</sup>, NEM1b, NEM2 and NEM3 are respectively AF480935, AF480937, AF480936 and AF480934.

A list of the accession numbers of reference sequences used in the phylogenetic analysis is available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org>).

adhering to the surface of *H. megidis* EU17 nematode IJs (Fig. 1). There is a level of specificity to this adherence; sporangia adhere to IJs of all *Heterorhabditis* spp. tested and of closely related parasites of the order Strongylida but not to any other soil nematodes tested (Enright *et al.*, 2001; M. R. Enright and C. T. Griffin, unpublished). Sporangia of closely related *Paenibacillus* species (eight species tested) did not adhere to *Heterorhabditis* spp. IJs (Enright *et al.*, 2001). The *Paenibacillus* sp. reproduces within the dead insect despite the *Photorhabdus*-produced antibiotics and emerging IJs can then carry large numbers of its sporangia out of the cadaver. IJs bearing sporangia then infect new insect hosts. Other *Heterorhabditis* spp. strains have also been found in similar association with endospore-forming bacteria. We noted the presence of bacteria in a *Heterorhabditis indica* strain from India contained in our collection and a *Heterorhabditis* sp. from Georgia, USA, was found associated with a bacterium initially identified as a *Bacillus* species (Marti & Timper, 1999). In both cases, the bacteria produced sporangia that were morphologically similar to those described by Enright *et al.* (2001). These bacteria–*Heterorhabditis* associations are recognizable by the tendency of the sporangia to ‘clump’ the nematodes in water (nematodes stick together in large cross-linked groups). In each of the cases mentioned, the nematodes were associated with the endospore-forming bacteria from the time that they were first isolated from soil (D. Marti and S. Easwaramoorthy, personal communication). In this paper, we have characterized four bacterial isolates that produce spindle-shaped sporangia and which were isolated from the *H. megidis*, *H. indica* and *Heterorhabditis* sp. strains



**Fig. 1.** Spindle-shaped *Paenibacillus* sp. sporangia adhering to the tail end of an *H. megidis* IJ. Bar, approx. 10  $\mu$ m.

mentioned above. A novel species, *Paenibacillus nematophilus* sp. nov., represented by two of these isolates, is proposed.

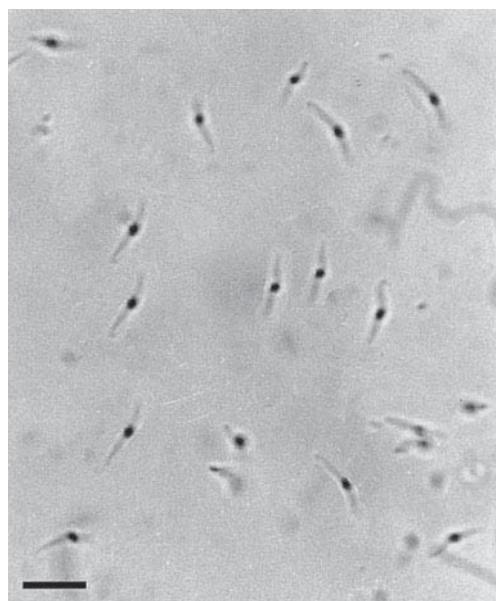
Bacteria were selectively isolated from *Heterorhabditis* nematodes based on resistance of their endospores to 0.4% hyamine (NEM1a<sup>T</sup>) or heat (all other isolates). Standard heat-isolation procedures were followed. Isolates were chosen based on production of spindle-shaped sporangia capable of clumping *Heterorhabditis* spp. IJs. All isolations were done on nutrient agar, following incubation at 30 °C. Standard phenotypic characterization of these isolates was carried out using the methods described by Gordon *et al.* (1973), Parry *et al.* (1983), Smibert & Krieg (1994) and Claus & Berkeley (1986), using appropriate *Paenibacillus* spp. controls. API tests (bioMérieux) were carried out as in Logan & Berkeley (1984), except that, with API 50CHB tests, results were also recorded after 4 days. All tests were replicated at least twice. Growth under anaerobic conditions was tested using an anaerobic jar with an AnaeroGen sachet (Oxoid) according to the manufacturer's instructions. Fatty acid analysis was carried out at the DSMZ (Braunschweig, Germany) using high-resolution GC and the profile was analysed using the Microbial Identification System (MIDI; Microbial ID) according to the manufacturer's instructions. Both the G + C content and DNA–DNA hybridization studies were also conducted at DSMZ. For these, DNA was isolated and purified according to the method of Cashion *et al.* (1977). In the G + C content studies, DNA was hydrolysed according to the method of Mesbah *et al.* (1989) and HPLC was performed as described by Tamaoka & Komagata (1984). Calibrations were done as described by Mesbah *et al.* (1989). DNA–DNA hybridization studies were carried out as described by De Ley *et al.* (1970), with the modifications described by Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were computed with the program TRANSFER.BAS (Jahnke, 1992).

Standard methods were used to extract bacterial genomic DNA before PCR amplification of almost the full-length 16S rRNA gene. Conserved eubacterial primers EB-27F (5'-AGAGTTTGATCMTGGCTCAG-3', M = A or C) and UN-1492R (5'-TACGGYTACCTTGTTACGACTT-3', Y = C or T) were used. PCR products were sequenced independently in both directions by Oswel Sequencing (Southampton, UK) using standard methods with an automated DNA sequencer and consensus sequences were determined. The 16S rRNA gene sequences of named *Paenibacillus* species and members of closely related genera were obtained from the GenBank database. All sequences were aligned using the program CLUSTAL X (Thompson *et al.*, 1997). Accession numbers of reference sequences obtained from GenBank are available as supplementary material in IJSEM Online (<http://ijs.sgmjournals.org>). Short sequence stretches at both ends of the alignment were excluded because of large numbers of ambiguous positions before pairwise percentage similarities of the sequences were determined using the program PAUP\* (Sinauer). The alignment was then manipulated manually, whereby positions of uncertain homology were excluded,

using the program SEAVIEW (Galtier *et al.*, 1996). Further phylogenetic analyses using distance, parsimony and maximum-likelihood criteria were carried out using the program PAUP\*. Confidence in phylogenetic hypotheses was assessed using the bootstrap ( $n=1000$ ) resampling method (Felsenstein, 1988) for distance and parsimony and quartet puzzling ( $n=1000$ ) (Strimmer & Von Haeseler, 1996) for maximum-likelihood.

Selective isolations from the various sporangia-associated nematode strains yielded numerous slow-growing bacterial isolates, all with similar colony morphology and all of which met the selection criteria employed. Four isolates, with at least one from each nematode strain, were chosen for further study. Isolates NEM1a<sup>T</sup> and NEM1b were isolated from *H. megidis* EU17 (from Estonia), while NEM2 and NEM3 were respectively isolated from *Heterorhabditis* sp. 'Line1' (from Georgia, USA) and *H. indica* LN2 (from India). Vegetative cells of these isolates stained Gram-negative (Gram-variable in older cultures) and were motile and rod-shaped. All four isolates produced morphologically similar spindle-shaped sporangia, in which an ellipsoidal endospore occupied a central/paracentral position, clearly swelling the sporangium (Fig. 2). Endospores were retained within the sporangia. Colonies of all isolates were thin, non-pigmented, regular and smooth with an undulate edge. All four of these nematode-associated isolates were phenotypically very similar (Table 1).

Phylogenetic analysis based on 16S rRNA gene sequences, using distance, parsimony and maximum-likelihood criteria, showed that the four nematode-associated isolates



**Fig. 2.** Mature sporangia of *Paenibacillus* sp. NEM1a<sup>T</sup> from a 5-week-old culture, showing that the endospores have been retained within the sporangia. Bar, approx. 10  $\mu$ m.

formed a phylogenetically distinct group within the genus *Paenibacillus*, separate from all other named species. This relationship was strongly supported by bootstrap and quartet-puzzling analyses. All four isolates had higher sequence similarity to each other (95.1–100%) than they had to any other named member of the genus (89.2–94%). Isolate NEM1a<sup>T</sup> shared 100% sequence similarity (across 1442 alignment positions) with the other isolate from the same nematode strain, NEM1b. However, similarities between these and the other isolates were lower. NEM1a<sup>T</sup> had 96.3% sequence similarity to NEM2 and 95.1% to NEM3, while NEM2 had 97.1% similarity to NEM3. These results are indicative of possible heterogeneity at the species level within the group (Stackebrandt & Goebel, 1994). Using the same alignment, *Paenibacillus macquariensis* was the named species with highest sequence identity to isolate NEM1a<sup>T</sup> (94%), followed by *Paenibacillus amylolyticus* and *Paenibacillus azoreducens* (93.6%), *Paenibacillus borealis* and *Paenibacillus durus* (93.5%) and *Paenibacillus macerans* (93.4%). Most similar to isolate NEM2 was *P. durus* (93.3%), while *P. amylolyticus* was most similar to isolate NEM3 (93.2%). Across a shorter alignment (966 alignment positions), *Paenibacillus illinoisensis* and *Paenibacillus chibensis* were also among the species with higher sequence similarity to the nematode-associated isolates. The genomic heterogeneity of the nematode-associated isolates suggested by the 16S rRNA sequences was confirmed by the results of DNA–DNA hybridization studies. Isolate NEM1a<sup>T</sup> shared 90.3% DNA–DNA similarity with NEM1b, but only 40.9 and 38.3% with isolates NEM2 and NEM3. Isolate NEM2 shared 60.4% DNA–DNA similarity with NEM3. In comparison, NEM1a<sup>T</sup> shared 25.8% DNA–DNA similarity with *P. azoreducens*, one of the species with highest 16S rRNA sequence identity to that isolate. According to the recommendations of Wayne *et al.* (1987), isolates should have similarity values of 70% or greater to be considered conspecific.

As is typical of *Paenibacillus* species (Ash *et al.*, 1993), the fatty acids of the nematode-associated isolates were mainly of the straight-chain saturated, anteiso- and iso-branched types, with anteiso-C<sub>15</sub> predominant, except in NEM2, where C<sub>16:0</sub> was slightly higher. Analysis yielded two major fatty acid types, namely, anteiso-C<sub>15</sub> and C<sub>16:0</sub> (Table 2). Straight-chain C<sub>14:0</sub> fatty acid was more common in the nematode-associated isolates than has been reported for most other *Paenibacillus* species (Shida *et al.*, 1997a, b; Nakamura *et al.*, 1996; Pettersson *et al.*, 1999; Elo *et al.*, 2001), an exception being *P. borealis* (Elo *et al.*, 2001). While all the isolates shared a similar overall pattern, there were some differences between them in the actual levels of some fatty acids. For example, whereas NEM1a<sup>T</sup> and NEM1b had similar levels of anteiso-C<sub>17</sub> (approx. 8%), NEM2 and NEM3 had higher levels (13 and 16%). Diagnostic amounts of C<sub>12:0</sub> and 3-hydroxy fatty acids were found in all of the nematode-associated isolates. These have normally been undetected or unreported for *Paenibacillus* species (Shida

**Table 1.** Differential characteristics of the four nematode-associated isolates and selected *Paenibacillus* species

Strains/species: 1, NEM1a<sup>T</sup>; 2, NEM1b; 3, NEM2; 4, NEM3; 5, *P. macquariensis* (data from Shida *et al.*, 1997a; Heyndrickx *et al.*, 1996b; Logan & Berkeley, 1984); 6, *P. amylolyticus* (Shida *et al.*, 1997b; Elo *et al.*, 2001); 7, *P. azoreducens* (Meehan *et al.*, 2001; this study); 8, *P. borealis* (Elo *et al.*, 2001; this study); 9, *P. macerans* (Shida *et al.*, 1997a; Logan & Berkeley, 1984; Elo *et al.*, 2001); 10, *P. illinoisensis* (Shida *et al.*, 1997b; Elo *et al.*, 2001); 11, *P. chibensis* (Shida *et al.*, 1997b; Elo *et al.*, 2001); 12, *P. durus* (Shida *et al.*, 1997a; Elo *et al.*, 2001); 13, *P. polymyxa* (Shida *et al.*, 1997a; Logan & Berkeley, 1984; Gordon *et al.*, 1973; this study). +, Positive; -, negative; v, variable; w+, weakly positive (acid produced more slowly than from other carbohydrates). NR, Not reported.

Characteristic	1	2	3	4	5	6	7	8	9	10	11	12	13
Endospore position*	C	C	C	C	T/ST	NR	T/ST	T/ST	T/ST	NR	NR	NR	C/ST
ONPG hydrolysis	-	-	-	-	+	+	NR	+	+	NR	NR	NR	+
Nitrate reduction	-	-	-	-	-	+	-	-	+	-	+	-	+
Production of:													
Acetylmethylcarbinol	+	+	+	+	-	-	-	-	-	-	-	+	+
Hydrolysis of:													
Casein	-	-	-	-	-	+	-	+	-	+	-	-	+
Starch	+	+	+	+	+	+	+	-	+	+	+	-	+
Aesculin	+	+	+	+	+	-	+	+	+	-	-	+	+
Gelatin	-	-	-	-	-	+	+	-	v	+	-	-	+
Litmus milk reaction	-	-	-	-	NR	-	+	+	+	+	-	NR	+
Gas from carbohydrates	-	-	-	-	-	-	NR	+	+	-	-	NR	+
Acid from:													
Lactose	-	-	-	-	+	-	+	+	+	-	-	-	+
L-Arabinose	-	-	-	-	-	+	-	+	+	+	+	+	+
D-Fructose	-	-	-	-	+	+	+	+	+	+	+	+	+
D-Galactose	-	-	-	-	+	+	+	+	+	+	+	+	+
D-Xylose	-	-	-	-	+	+	+	+	+	+	+	-	+
Glycerol	-	-	-	-	-	+	+	+	+	+	-	-	+
D-Mannitol	-	-	-	-	+	+	+	+	+	+	+	-	+
D-Ribose	-	-	+	+	+	+	+	-	+	+	+	-	+
D-Mannose	+	-	+	-	v	+	+	+	+	+	-	+	+
Melibiose	-	-	-	-	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	-	+	-	-	v	+	+	-	-
Melezitose	-	-	-	-	-	+	+	+	+	+	+	+	v
D-Raffinose	-	-	-	-	+	-	+	+	+	+	-	+	+
Inulin	-	-	-	-	-	+	+	+	+	+	+	+	v
5-Keto-D-gluconate	w+	w+	w+	w+	-	-	-	-	v	-	-	-	v
DNA G+C content (mol%)	44.0	44.2	44.4	43.9	39	46	47	54	53-54	48	53	50	43-46

\*C, Central/paracentral; T, terminal; ST, subterminal.

*et al.*, 1997a, b; Nakamura *et al.*, 1996; Elo *et al.*, 2001; Heyndrickx *et al.*, 1996a; Meehan *et al.*, 2001).

All of the nematode-associated isolates shared a high degree of phenotypic similarity and conformed to the description of the genus (Shida *et al.*, 1997a). They could easily be distinguished from closely related species on the basis of numerous differential characteristics (Table 1). For example, the endospore was always located in a central/paracentral position, while a terminal/subterminal position is the most common in their closest relatives. Strains of some related *Paenibacillus* can have both. For example, while all *Paenibacillus polymyxa* strains produce terminal/sub-terminal endospores, some also produce sporangia with

central/paracentral endospores (Logan & Berkeley, 1984). Endospores of the nematode-associated isolates are retained within the sporangium, even following long periods of storage. This is not the case with the majority of other described *Paenibacillus* species (N. Logan, personal communication). All the nematode-associated isolates had distinct profiles in API tests, easily distinguishable from those of closely related species. In API 50CHB tests, NEM1a<sup>T</sup> utilized 16 carbohydrate substrates out of 49, while NEM1b utilized 15, NEM2 utilized 17 and NEM3 utilized 16, with 15 of these being common to all four isolates. These numbers are low compared with those found for other *Paenibacillus* species in both this and previous studies (Logan & Berkeley, 1984; Elo *et al.*, 2001). Incubation of the API 50CHB tests for

**Table 2.** Fatty acid composition of *Heterorhabditis*-associated *Paenibacillus* isolates and other *Paenibacillus* species

Values are percentages of total fatty acids. Strains: 1, NEM1a<sup>T</sup>; 2, NEM1b; 3, NEM2; 4, NEM3; 5, *P. borealis* DSM 13188<sup>T</sup> (data from Elo *et al.*, 2001); 6, *P. azoreducens* DSM 13822<sup>T</sup> (Meehan *et al.*, 2001); 7, *P. chibensis* NRRL B-142<sup>T</sup> (Shida *et al.*, 1997b); 8, *P. amylolyticus* NRRL NRS-290<sup>T</sup> (Shida *et al.*, 1997b); 9, *P. macquariensis* CIP 103269<sup>T</sup> (Shida *et al.*, 1997a); 10, *P. macerans* JCM 2500<sup>T</sup> (Shida *et al.*, 1997b). NR, Not reported; ND, not detected.

Fatty acid	1	2	3	4	5	6	7	8	9	10
<b>Saturated</b>										
C <sub>12:0</sub>	1.80	2.37	2.30	2.13	NR	NR	NR	NR	ND	ND
C <sub>14:0</sub>	15.50	14.53	16.87	8.74	18.6	3.5	0.3	0.6	0.7	3.7
C <sub>15:0</sub>	0.91	1.08	0.54	0.39	<0.2	0.1	0.6	0.5	1.2	0.5
C <sub>16:0</sub>	22.71	20.17	22.35	17.41	10.0	22.1	5.3	20.1	2.9	17.9
Iso C <sub>14:0</sub>	1.79	1.90	2.06	1.08	4.9	0.8	0.8	0.2	0.8	1.8
Iso C <sub>15:0</sub>	1.35	1.52	2.08	3.06	11.5	5.9	4.0	2.9	5.2	2.6
Iso C <sub>16:0</sub>	13.22	12.62	14.77	11.2	10.5	8.6	12.2	4.1	2.6	17.1
Iso C <sub>17:0</sub>	1.38	1.25	2.12	3.33	4.9	5.6	3.2	5.8	0.4	4.1
Anteiso C <sub>15:0</sub>	27.66	31.17	19.09	30.71	35.2	33.9	57.8	36.9	81.0	34.5
Anteiso C <sub>17:0</sub>	8.25	7.51	12.99	16.29	2.1	19.8	14.3	11.5	0.7	16.1
<b>Others</b>										
C <sub>16:1</sub> ω11	ND	ND	ND	ND	1.3	ND	ND	0.5	0.6	0.1
Iso C <sub>16:0</sub> 3-OH	1.22	1.37	0.34	0.64	NR	NR	NR	NR	NR	NR
C <sub>17:0</sub> 2-OH	1.5	1.61	0.99	2.14	NR	NR	NR	NR	NR	NR

2 days longer than described in Logan & Berkeley (1984) resulted in positive results for utilization of 5-keto-D-gluconate by the nematode-associated isolates. This was not the case with control *Paenibacillus* species.

Differentiation of the nematode-associated isolates from *P. macquariensis*, the species with highest 16S rRNA gene sequence identity to NEM1a<sup>T</sup>, is based on the position of the endospore within the sporangium, their inability to grow at 5 °C and their Voges–Proskauer reaction, as well as their inability to utilize a wide range of carbohydrates (Table 1).

The nematode-associated isolates differed somewhat in the conditions under which growth occurred. All four isolates grew at between 15 and 37 °C. However, only NEM1a<sup>T</sup> and NEM1b grew at 10 °C. This finding may be linked to climatic differences between the geographical origins of the different isolates. None of the isolates grew at 5 or 40 °C. Isolates NEM1a<sup>T</sup>, NEM1b and NEM3 grew at pH 6–11, whereas NEM2 grew at pH 6–10. Isolates NEM1a<sup>T</sup> and NEM1b grew in 2% (w/v) NaCl, but not in 3%, whereas NEM2 and NEM3 grew in 3% (w/v) NaCl but not in 4%.

Despite the phenotypic and ecological similarities, genomic heterogeneity exists within this group of isolates, such that its members are likely to represent more than one species. Certain characteristics (growth temperatures, differential ability to tolerate NaCl during growth, ability to utilize D-ribose and features of their fatty acid profiles) separate one group, NEM1a<sup>T</sup> and NEM1b, from NEM2 and NEM3, but there is little to separate the latter two isolates. Strain NEM1a<sup>T</sup> grew *in vivo* (in infected insect hosts) with some, but not all *Heterorhabditis* spp. tested, while NEM2 and

NEM3 grew with a similar spectrum of *Heterorhabditis* spp., but different from that of NEM1a<sup>T</sup> (M. R. Enright and C. T. Griffin, unpublished). Here, we propose isolates NEM1a<sup>T</sup> and NEM1b to represent a single species, *Paenibacillus nematophilus* sp. nov., with NEM1a<sup>T</sup> as the type strain. While NEM2 and NEM3 are phylogenetically very closely related to this proposed species, it is clear that they themselves are representative of at least one, but most likely two, novel species. To date, bacteria with these characteristics have been recovered only rarely fortuitously during the isolation of entomopathogenic nematodes from soil and the isolates studied here have been maintained in laboratory culture with nematodes for up to 10 years. Examination of further isolates from the wild (nematode-associated or otherwise) and of more phenotypic characters would be desirable so that the true extent of variation within these remaining potential species can be explored.

**Description of *Paenibacillus nematophilus* sp. nov.**

*Paenibacillus nematophilus* (ne.ma.to'phi.lus. N.L. n. *nematoda* nematode; Gr. adj. *philos* loving or having affinity for; N.L. adj. *nematophilus* nematode-loving).

Cells are rod-shaped, motile, 3.5–7.0 μm long and 0.5–1.0 μm wide. Cells stain Gram-negative (Gram-variable in older cultures). Oval-shaped endospores are produced in swollen, spindle-shaped sporangia and lie in a central/paracentral position. Endospore is retained within the sporangium. Endospores measure on average 1.7 × 1.2 μm, while sporangia are 6.5–10.6 μm long. Relatively slow-growing on nutrient agar, with colonies measuring

0.5–4.0 mm in diameter. Colonies are thin, non-pigmented, regular, smooth and slightly umbonate with an undulate edge. Catalase-positive and oxidase-negative. Under anaerobic conditions, growth is weak and germination of endospores is poor compared with aerobic conditions. Grows in 2% but not 3% (w/v) NaCl. Grows optimally at 30 °C. Growth occurs at between 10 and 37 °C. No growth at 5 or 40 °C. Growth at pH 6–11; no growth at pH 5.6. As determined with API 20E strips,  $\beta$ -galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, tryptophan deaminase and urease are not produced. Citrate is not utilized. Hydrogen sulfide, indole and NO<sub>2</sub> are not produced. Gelatin is not hydrolysed. The Voges–Proskauer reaction is positive. Starch is hydrolysed. Casein and Tween 80 are not hydrolysed. Isolates are negative for egg yolk lecithinase activity. No colour change in litmus milk. In API 50CHB galleries, when API CHB suspension medium is used, aesculin is hydrolysed and acid but no gas is produced from D-glucose, methyl  $\alpha$ -D-glucoside, N-acetylglucosamine, amygdalin, arbutin, salicin, cellobiose, maltose, sucrose, trehalose, starch, glycogen and gentiobiose. Acid is produced at a slower rate from 5-keto-D-gluconate. Utilization of D-mannose is variable (type strain is positive). Glycerol, ribose, erythritol, D-arabinose, L-arabinose, D-xylose, L-xylose, adonitol, methyl  $\alpha$ -D-xyloside, galactose, fructose, sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl  $\alpha$ -D-mannoside, lactose, melibiose, inulin, melezitose, raffinose, xylitol, D-turanose, D-lyxose, D-tagatose, D-fucose, L-fucose, D-arabitol, L-arabitol, gluconate and 2-ketogluconate can not be utilized as sole carbon sources. The major fatty acids are anteiso-C<sub>15</sub> and C<sub>16:0</sub> and the G + C content is approximately 44 mol% as determined by HPLC. The type strain, strain NEM1a<sup>T</sup> (=DSM 13559<sup>T</sup>=NCIMB 13845<sup>T</sup>), and strain NEM1b were found associated with the entomopathogenic nematode *H. megidis* EU17 isolated from Estonia.

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